Inhibition of *Clostridium botulinum* by 5-Nitrothiazoles

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A number of 5-nitrothiazoles with various substituents in the 2-position were tested for inhibition of *Clostridium botulinum* in a culture medium. Thiazole itself or 2-bromo- or 2-methylthiazole at 30 μg/ml did not inhibit the organism. An amino group in the 2-position of thiazole inhibited at 10 μg/ml. Substitution of a nitro group in the 5-position of 2-aminothiazole increased the inhibitory level to 0.12 μg/ml; acetyl-, propionyl-, or butyroyl-2-amino-5-nitrothiazole inhibited at 0.04 μg/ml. Benzoyl-2-amino-5-nitrothiazole inhibited at 0.16 μg/ml; this increased to 0.01 μg/ml when the benzoyl group carried a nitro group in the *m*- or *p*-position; a nitro group in the *o*-position, on the other hand, inhibited at 0.04 μg/ml. Unsaturated aliphatic acyls decreased inhibition. The greatest activity was exhibited by 2-nonanoyl- and 2-lauroylamides, with minimum inhibitory concentrations of 0.005 and 0.0025 μg/ml, respectively.

Nitroheterocyclic compounds have been shown to possess considerable antimicrobial activity. Cavalleri et al. (4) demonstrated broad-spectrum in vitro antimicrobial activity by 1-methyl-2-nitro-5-vinylimidazole and 1-methyl-2-nitroimidazole-5-carboxaldehyde. Asato and Berkelhammer (2) indicated that 2-amino-5-(1-methyl-2-nitro-5-imidazolyl)-1,3,4-thiadiazole and 2-amino-5-(1-methyl-2-nitro-5-imidazolyl)-1,3,4-oxadiazole were bactericidal. Weuffen et al. (14) indicated that tetrahydro-1,3,5-thiadiazine-2-thiones were active against gram-positive and gram-negative bacteria as well as against *Trichophyton microsporon, Trichosporon cutaneum,* and *Candida albicans*. 5-Nitrofuraldehydes were shown by Weuffen et al. (16) to have bacteriostatic and fungistatic activity, whereas 5-nitro-2-vinylfurans with various structural units were found by Fujimoto (7) to possess in vitro activity against gram-negative bacteria. Furfurylidinethiazoles are active intestinal disinfectants, fungicides, and parasiticides (Etaslissements Clin. Byla, French Patent 1,604,530, June 1967; CA. 79, 32038r).

Gibbs and Robinson (8) demonstrated inhibition of *Streptococcus* and *Staphylococcus* by 10 μg of 2-mercapto-5-(2-acetoxyethyl)-4-methylthiazole, 2-mercapto-5-(2-hydroxyethyl)-4-methylthiazole, and 2-amino-5-mercapto-4-methylthiazole·HCl per ml. Weuffen et al. (15) studied the effect of 33 thiazole derivatives on the growth of *Staphylococcus aureus* and several gram-negative bacteria and found 6-(*p*-methoxyphenyl)-imidazole[2,1-b]thiazole effective, whereas aminobenzothiazole derivatives, thiadiazoles, and sulfathiazole were ineffective.

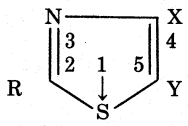
Castro and Navarro (U.S. Patent 3,821,390, June 1974; CA. 81, 163570x) used 2-acetylamino-5-nitrothiazole for curing hemorrhagic colitis in swine and preventing dysentery. This compound was also effective against experimental mouse infections of *Trypanosoma cruzi* (3). *Histomonas meleagridis* was inhibited in vitro by 5 μg of 2-amino-5-nitrothiazole and 2.5 μg of 2-acetamido-5-nitrothiazole per ml (10); the effect appeared to be on the associated bacteria rather than on the protozoon itself. Islip et al. (12) indicated that 5-nitrothiazoles were schistosomicidal in experimentally infected rhesus monkeys. Cuckler et al. (5) showed 2-acetamido-5-nitrothiazole to be also capable of protecting mice against experimental protozoal infections, including *Trichomonas faetus* and *T. vaginalis*. Kolosova et al. (13) also showed that 2-amino-5-nitrothiazole was effective in vitro against *T. vaginalis;* the acyl derivatives were even more active. Hall et al. (9) demonstrated the effectiveness of this class of compounds in treating histomoniasis (blackhead) in turkeys.

In previous unpublished work, one of us (C.N.H.) had shown that 5-nitrothiazole inhibited *Clostridium sporogenes*. The in vitro activity of 5-nitrothiazoles against *C. botulinum* was investigated for possible use as a nitrite substitute for inhibiting clostridia in cured meat products. They are low in toxicity to mice, chickens, turkeys, and dogs (5) and are currently approved for animal feed use for prevention and control of turkey blackhead (1).

MATERIALS AND METHODS

Thiazole derivatives. The compounds tested are listed in Table 1. Compounds no. 14 through 30 were

TABLE 1. *Effect on C. botulinum inhibition of the groups substituted in 2- and 5-positions of thiazole*



No.	R	X	Y	MIC (μg/ml)
1	H	H	H	>20
2	H	H	NO_2	0.90
3	Br	H	H	>20
4	Br	H	NO_2	>20
5	CH_3	H	H	>20
6	CH_3	H	NO_2	8
7	NH_2	H	H	>20
8	NH_2	H	Cl	>20
9	NH_2	H	Br	>20
10	NH_2	CH_3	H	>20
11	NH_2	H	CH_3	>20
12	NH_2	H	NO_2	0.12
13	NH_2	H	p-$NO_2 \cdot C_6H_4SO_2NH$	>20
14	Acetamido	H	NO_2	0.04
15	n-Propionylamido	H	NO_2	0.04
16	β-Bromopropionylamido	H	NO_2	2.50
17	Methoxyacetamido	H	NO_2	0.08
18	n-Butyroylamido	H	NO_2	0.04
19	n-Nonanoylamido	H	NO_2	0.005
20	n-Lauroylamido	H	NO_2	0.0025
21	Crotonoylamido	H	NO_2	0.30
22	Sorboylamido	H	NO_2	0.30
23	Benzoylamido	H	NO_2	0.16
24	Nicotinoylamido	H	NO_2	0.16
25	p-Chlorobenzamido	H	NO_2	0.08
26	p-Hydroxybenzamido	H	NO_2	0.08
27	p-Methoxybenzamido	H	NO_2	0.16
28	o-Nitrobenzamido	H	NO_2	0.04
29	m-Nitrobenzamido	H	NO_2	0.01
30	p-Nitrobenzamido	H	NO_2	0.01
31	3-Nitrophthalimido	H	NO_2	0.04
32	3-Nitrophthalamido	H	NO_2	0.60
33	3,4,5,6-Tetrachlorophthalamido	H	NO_2	0.16
34	3,4,5,6-Tetrachlorophthalimido	H	NO_2	0.02
35	Norbornene-5-carboxamido	H	NO_2	0.60

prepared in this laboratory by acylation of 2-amino-5-nitrothiazole. The reaction mixture was 200 ml of ethyl acetate, 0.05 mol of triethylamine, and 0.05 mol of 2-amino-5-nitrothiazole in a 500-ml three-necked reaction flask equipped with a condenser, stirrer, and addition funnel, mounted in an oil bath controlled at about 40°C. To this was added, dropwise with stirring, 0.05 mol of the appropriate acyl chloride in 100 ml of ethyl acetate. Within about 1 h the addition was completed, and the reaction mixture was allowed to react further for 2 h. The precipitate of triethylamine hydrochloride was filtered and washed with 100 ml of ethyl acetate. The combined filtrates were then concentrated in vacuo at 25°C/1 mm until a dry residue was obtained; this produced nearly quantitative yields of crude 2-acylamido-5-nitrothiazoles. These were purified by recrystallization from toluene (about 20 ml/g), dried in vacuo at 56°C/0.1 mm, and analyzed. Their identity was confirmed by infrared and mass spectra. The melting points of compounds no. 14 through 30 were as follows, citing only the acyls: acetyl, 269 to 702°C; propionyl, 195 to 297°C; β-bromopropionyl, 175 to 177°C; methoxyacetyl, 135 to 137°C; n-butyroyl, 178 to 179°C; n-nonanoyl, 138 to 140°C; n-lauroyl, 130 to 132°C; crotonoyl, 218 to 219°C; sorboyl, 175 to 177°C; benzoyl, 259 to 260°C; nicotinoyl, 190°C (decomposition); p-chlorobenzoyl, 268 to 269°C; p-hydroxybenzoyl, 172 to 173°C; p-methoxybenzoyl, 234 to 235°C; o-nitrobenzoyl, 244 to 245°C; m-nitrobenzoyl, 195 to 197°C; and p-nitrobenzoyl, 173 to 174°C.

Five compounds (no. 31 through 35) were obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Silver Spring, Md. The remainder of the compounds (no. 1 through 13) were obtained from Aldrich

Chemical Co. Commercial compounds were used directly, without further purification. All these compounds possessed a minimum purity of 97%, as established by analyses and spectral studies.

Assay method. The assay method of Huhtanen (11) was used with 1-mg/ml ethanol solutions of the compounds. The lowest dilution used was 1:50 (0.1 ml of solution to 5 ml of medium) since ethanol was inhibitory to *C. botulinum* at dilutions of 1:25 or less. The highest minimum inhibitory concentration (MIC) possible was thus 20 μg/ml. Ethanol was used because some of the compounds were water insoluble; it also acted as a sterilizer and eliminated the possibility of heat inactivation by autoclaving or adsorption on filter membranes.

RESULTS

The effect of thiazole and its derivatives on inhibition of *C. botulinum* is shown in Table 1. Thiazole and 2-amino-, 2-bromo-, and 2-methylthiazole had an MIC greater than 20 μg/ml; substitution in the 5-position with bromo, chloro, methyl, and nitro groups did not increase the MIC. A significant increase in activity was observed when thiazole was substituted in the 2-position with an amino group and in the 5-position with a nitro group. Thus, 2-amino-5-nitrothiazole gave an MIC of 0.12 μg/ml. Further increase in activity was obtained by acylation of the amino group; 2-acetyl-, 2-propionyl-, and 2-butyroylamino-5-nitrothiazole all exhibited the same activity, with an MIC of 0.04 μg/ml. The most potent activity was exhibited by 2-nonanoyl- and 2-lauroylamido-5-nitrothiazole, with MICs of 0.005 and 0.0025 μg/ml, respectively. Unsaturation of the acylating group lowered the activity; 2-crotonoyl- and 2-sorboylamino-5-nitrothiazole showed an MIC of 0.30 μg/ml. There was a further decrease in activity with 2-(β-bromopropionyl)amino-5-nitrothiazole (MIC = 2.5 μg/ml). 2-Benzoyl- and 2-nicotinoylamido-5-nitrothiazole exhibited about the same activity as the unsaturated acyls (0.16 μg/ml). Substitution in the benzoyl moiety caused an increase of the activity. Thus 2-(*p*-chlorobenzoyl)amino- and 2-(*p*-hydroxybenzoyl)amino-5-nitrothiazole were twice as active as the benzoyl compound itself. Potent activity (MIC = 0.01 μg/ml) was observed when a nitro group was substituted in the *m*- and *p*-positions of the benzoyl; however, *o*-substitution activity gave an MIC of 0.04 μg/ml.

Other compounds tested. We also determined the MICs of a number of other compounds: thiazolidine, 1-thiazolidine-4-carboxylic acid, 2-amino-2-thiazoline, 2-amino-4-imino-2-thiazoline hydrochloride, 3-nitro-L-tyrosine, and 3,4-dihydroxyphenylalanine (L-DOPA). None was active (MIC > 20 μg/ml).

DISCUSSION

Most of the interest concerning the biological activity of the thiazoles has centered on their protozoocidal rather than bactericidal effects. Kolosova et al. (13) found *T. vaginalis* to be inhibited by a 10^{-3} dilution of 2-aminothiazole; this decreased to 4.5×10^{-5} when a 5-nitro group was added and to 10^{-6} with 2-acetamido-5-nitrothiazole. Our results with *C. botulinum* indicated a similar order of activity, with an MIC of >20 μg/ml exhibited by 2-aminothiazole, 0.12 μg/ml by 2-amino-5-nitrothiazole, and 0.04 μg/ml by 2-acetamido-5-nitrothiazole. Our results further indicated that, although acetyl, propionyl, and butyroylamino-5-nitrothiazoles were of equivalent activity, their homologs, nonanoyl- and lauroylamide, were found to be about 10 times as active, whereas a benzoyl group resulted in decreased inhibition. Substitution of a nitro group in the *m*- or *p*-position greatly increased activity; *o*-nitro also increased activity, but not as much as the *m*- or *p*-nitro.

Cuckler et al. (5) also showed that *T. faetus* was inhibited equally by 2-acetyl-, 2-propionyl-, or 2-butyroylamino-5-nitrothiazole; all were more effective than 2-amino-5-nitrothiazole. It is possible that the inhibition of *T. vaginalis* and *H. meleagridis* by these compounds is a result of the inhibition of associated symbiotic bacteria rather than of the protozoa per se. Horton-Smith and Long (10) suggested that furazolidone acts against *H. meleagridis* in this manner. Previous unpublished observations by one of us (C.N.H.) on the growth of *H. meleagridis* in vitro indicated that the organism could not be grown bacteria-free; inhibition of the protozoon occurred with 2-acetamido-5-nitrothiazole as well as with antibiotics.

Woolford et al. (17) indicated that bovine serum albumin and myosin react with nitrite, producing 3-nitrotyrosine and 3,4-dihydroxyphenylalanine as well as other compounds. These were not effective inhibitors of *C. botulinum* in our system (MIC > 20 μg/ml).

The relation between the physicochemical properties and physiological activity against *Trichomonas* of 2-amino-, 2-acetylamino-, 2-propionylamino-, 2-butyroylamino-, and 2-valeroylamino-5-nitrothiazoles were studied by Danek et al. (6), using ultraviolet light, a visible spectrum, and pK_a. Acylation of the amino group resulted in a decrease of the pK_a values, thus increasing the acidity. There was no difference in the observed pK_a values with acyls from C_2 to C_6. Our results indicate that inhibition of *C. botulinum* was similar with C_2, C_3, or C_4 acyl groups; however, a decrease in the ac-

tivity to one-fourth that of the 2-amino-5-nitrothiazole was noted with a benzoyl or nicotinoyl group. This indicates that the acid character is of some importance in determining antimicrobial activity. Unsaturation in the chain also appeared to reduce the activity, whereas substitution of a nitro group in the *m*- or *p*-position of the benzoyl increased the activity fourfold over that of the saturated aliphatic acyls.

A general assumption could be made that all the 2-*n*-acylamido-5-nitrothiazoles possess the same pK_a values (6); however, the 10-fold-higher activity of nonanoyl- and lauroylamido-5-nitrothiazole indicates that there is no simple relationship between the pK_a values and anticlostridial activity. Unfortunately, these two compounds possess very low solubility in aqueous media between pH 2 and 10, and we were not able to determine their pK_a values by spectroscopic procedures (6). Similar difficulties were encountered in dealing with the aromatic amides.

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